Synthetic Culture of Mycorrhizae Of Southern Pines

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IDENTIFICATION of the fungal complement of mycorrhizae of forest trees is necessary before exhaustive studies on the relationship between the two symbionts can be made. Ideally, the fungus should be isolated directly from the mycorrhiza as collected in the field and identified. This, unfortunately, has seldom proved feasible, but new techniques are giving encouraging results. First, attempts to isolate the organism in pure culture either from soil or from the roots themselves have generally failed even though living fungal hyphae can be readily observed in fresh mycorrhizae. And, second, mycelium alone usually is not sufficient to identify the fungal partner. A sporophore is needed for positive determination, but fruiting has proved difficult to induce artificially with some fungi, and has not been obtained at all with most.

These obstacles were in large part circumvented by the development of the pure culture technique for mycorrhizal synthesis by Melin (1921). His method is now standard for ascertaining which fungi are capable of forming mycorrhizae when associated with roots of forest trees. A seedling is germinated aseptically and planted in a sterile nutrient medium to which inoculum of the suspected mycorrhiza-forming fungus is added. After 3 or 4 months roots are examined for the presence of mycorrhizae.

In the United States, Doak (1934b) employed this technique to determine the mycorrhiza-forming capacity of a number of fungi with several species of pine. He obtained typical ectotrophic mycorrhizae with the following combination of fungi and roots of pine seedlings: *Boletus bicolor* Peck with *Pinus rigida* Mill.; *B. brevipes* Peck with P. *rigida* and P. *taeda* L; B. *chromapes* Frost. with *P. taeda; Boletinus pictus* Peck with *P. strobus* L., *P. taeda,* P.*resinosa* Ait., and P. *rigida*; *Cantharellus cibarius* Fr. with *P. rigida, P. taeda,* and *P. strobus; Russula lepida* Fr. with P. *rigida,* P. *taeda,* and P. *strobus*; and *Scleroderma vulgare* (Horn.) Fr. with *P. strobus.*

Mycorrhizal formation by *Lepiota rhacodes* (Vitt.) Quél. with roots of *P. virginiana* Mill. was demonstrated similarly by Hacskaylo (1953). More recently Hacskaylo and Palmer (1955) reported the formation of ectotrophic mycorrhizae on *P. virginiana* by *Amanita caesaria* Schw., *A. frostiana* Peck, *A. rubescens* S. F. Gray, *B. bicolor* Peck, *B. variegatus* Fr., and *Rhizopogon roseolus* (Corda) Th. Fr.

The pure culture technique for mycorrhizal synthesis was employed in the present study to identify fungi entering into symbiotic relationship with roots of the four major southern pine species—shortleaf

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pine (*Pinus echinata* Mill.), loblolly pine (*P. taeda* L.), slash pine (*P. elliottii* Engelm. var. *elliottii*), and longleaf pine (*P. palustris* Mill.). This report represents part of a broad investigation of mycorrhizae of these species aimed at understanding their relationship to root pathogens and other organisms in the rhizosphere and to nutrition.

Materials and Methods

Only species of Hymenomycetes collected in the Piedmont and Coastal Plain regions of the Southeast were utilized in the present study. Both pine and pine-hardwood stands containing one or more of the test pine species, shortleaf, loblolly, slash, and longleaf pines, were canvassed. The most common genus encountered was *Boletus* followed by *Russula*, *Amanita*, and *Lactarius*. A species of *Clitocybe*, *C. laccata* (Scop.) Quél., has been collected in pine stands from western North Carolina to northern Florida, fresh sporophores being found throughout the year in the latter area.

Pure cultures of test fungi were obtained by removing small pieces of tissue from the context of sporophores and planting on malt extract and Hagem[1] agar media. Only about 5 percent of the isolations attempted are being successfully grown in pure culture. Some species showed improved growth when pine or hardwood-humus extract was added to the basic malt-extract medium. This was not correlated with their respective habitats. Addition of 25 ppm of thiamin to malt-extract agar appeared to aid growth of several species. Streptomycin incorporated in both malt extract and Hagem agar, at the rate of 80 ppm, was helpful in suppressing bacteria.

The following schedule was used to obtain sterile pine seedlings. Seed were first washed in detergent solution for 5 minutes, then rinsed in tap water. Next followed treatment in 1:500 mercuric chloride solution. Shortleaf pine seed were sterilized for 5 minutes; loblolly and slash pine seed for 12 minutes; and, longleaf pine seed were first hulled and then sterilized for 15 seconds. Thorough rinsing of seed in several changes of sterile water followed. The seed were then planted on cornmeal agar and placed in the refrigerator at 6° C for two weeks to break dormancy. After refrigeration, the planted seed were germinated at room temperature.

Sterile, germinating seedlings were removed from the agar and planted in 2-liter Erlenmeyer flasks containing vermiculite medium, as recommended by Hacskaylo (1953), to which nutrient solution formulated by Melin (1921) and modified by Norkrans (1949) had been added. At the same time, or shortly thereafter, a bit of inoculum from a pure culture of the test fungus was planted in the vermiculite about 1 inch from the seedling. The flasks were held in the laboratory under artificial light for 10 days to confirm the absence of contamination, after which they were placed in a greenhouse fitted with 30 percent overhead slat shading. The bases of the flasks were immersed in a water bath maintained at 17° C to prevent excessive heating of the medium and the interior of the flask. On the hottest summer days the temperature of the vermiculite did not exceed 24° C.

After 3 to 4 months, the flask was opened, and a bit of vermiculite medium placed on nutrient agar to reisolate the fungus and to verify the purity of the culture. The seedling, now 5 to 7 inches tall and bearing secondary needles, was removed and the roots carefully examined for the presence and degree of development of mycorrhizae. Mycorrhizal root tips were severed and placed in weak chrom-acetic killing solution in preparation for later sectioning and microscopic examination to confirm the presence or absence of hyphal penetration.

Material for sectioning was dehydrated using Johansen's (1935) tertiary butyl al-

[1]Formula obtained by personal communication in 1959 from E. Hacskaylo, Plant physiologist, Forest Service, U.S. Department of Agriculture, Beltsville, Maryland.

cohol method. Sections were cut 12 microns thick. Satisfactory differential staining was obtained using ordinary safranin fast-green combinations, the orseillin BB and crystal violet schedule of Doak (1934a), and the safranin-picro-aniline blue method described by Jackson (1947).

Results and Discussion

The fungi thus far tested and results obtained with shortleaf, loblolly, slash, and longleaf pines are shown in Table 1. Synthesis of bifurcate, ectotrophic mycorrhizae with good mantle and Hartig net development on roots of all four pine species was demonstrated for *Clitocybe laccata*. Figure 1 illustrates typical hyphal mantle and Hartig net development on mycorrhizae of slash pine produced by *C. laccata* in flask culture. In all positive syntheses observed, the Hartig net hyphae developed between all cortical cells. Positive synthesis was also

TABLE 1. Results of synthetic culture of pine mycorrhizae.

Fungus	Pine species[1]				Description of mycorrhizae		
	Shortleaf	Loblolly	Slash	Longleaf	Form and type	Mantle development	Hartig net development
Amanita muscaria (L.-Pers.) Quél.	I	+	I	I	Bifurcate, ectotrophic	Good	Good
Boletus betula Schw.	—	—	+	—	Coralloid, bifurcate, ectotrophic	Good	Good
B. communis Bull.	I	+	+	—	Bifurcate, ectotrophic	Good	Good
B. rubropunctus Peck	—	—	—	I			
B. spp. (Florida)	I	+	+	+	Coralloid, bifurcate, ectotrophic	Good	Good
Clitocybe laccata (Scop.) Quél.	+	+	+	+	Bifurcate, ectotrophic	Good	Good
C. piceina Peck	+	I	I	I	Bifurcate, ectotrophic	Fair	Fair
C. tabescens (Scop.) Bres.	—	—	—	—			
Cortinarius albidipes Peck	I	—	—	I			
Hypholoma spp.	—	—	I	I			
Lactarius spp. (Florida)	+	—	—	—	Bifurcate, ectotrophic	Good	Good
Lycoperdon spp.	I	—	—	—			
Pisolithus tinctorius (Mich. ex Pers.) Coker and Couch	+	—	—	I	Coralloid, bifurcate, ectotrophic	Good	Good
Psalliota campestris (L.) Quél.	I	—	—	I			

[1]I, test incomplete; +, mycorrhizae formed; —, mycorrhizae not formed.

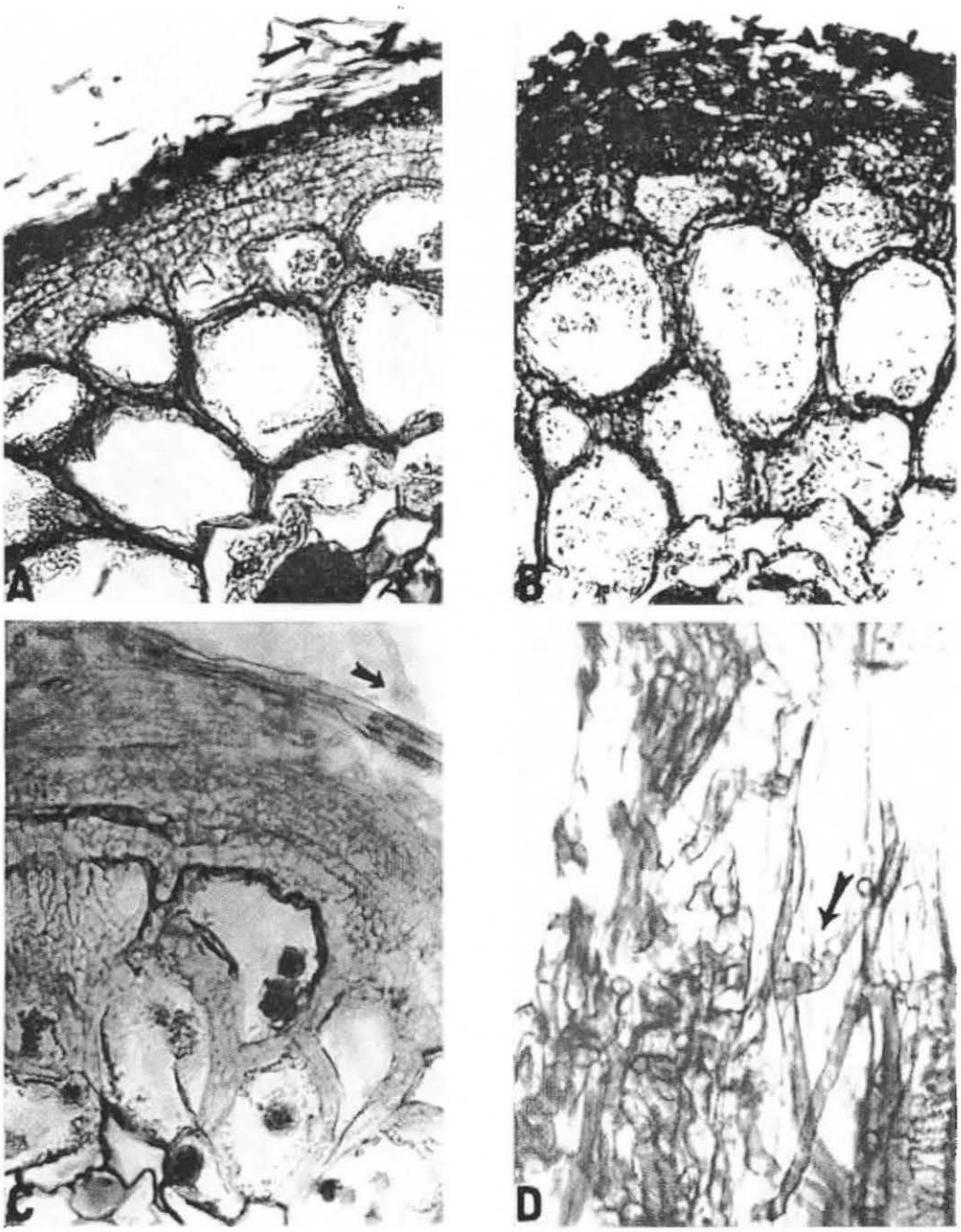

FIGURE 1. *Photomicrographs of sections of mycorrhizae produced on slash pine with* Clitocybe laccata. *Transverse sections in A* (× *3075*), *B* (× *3225*), *and C* (× *3375*) *show well-developed fungus mantle and intercellular Hartig net. D, Longitudinal section of mantle* (× *4725*). *Arrows point to clamp connections in mantle hyphae.*

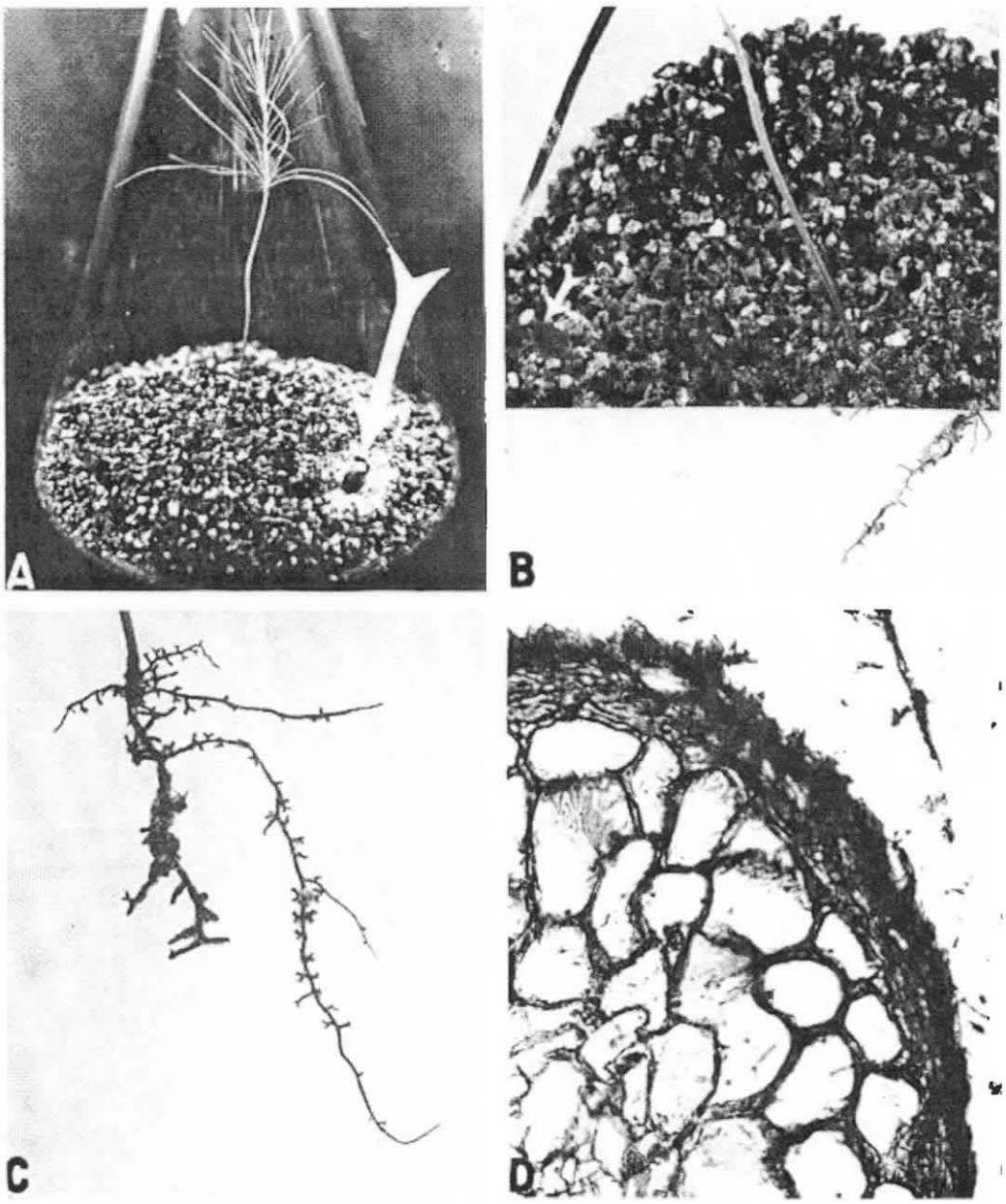

FIGURE 2. *Cultural and histological confirmation of mycorrhizal synthesis by* Boletus communis *on slash pine. A, Seedling growing in test flask—arrow points to sporophore of* B. communis *produced in the flask 52 days after inoculation. B, Test flask broken open to allow closeup view of sporophore (left, at arrow), connected by rhizomorphs to mycorrhizal root at right. C, Complete root system of test seedling, showing mycorrhizae and heavy mycelial envelopment. D, Transverse section of mycorrhiza from test seedling, with fungus mantle and intercellular Hartig net development* (× 2870).

achieved with the following: *Amanita muscaria* with loblolly pine; *Boletus betula* with slash pine; *B. communis* with loblolly and slash pine; *B.* spp. (unidentified, from northern Florida) with loblolly, slash, and longleaf pine; *Pisolithus tinctorius* with shortleaf pine; and *Lactarius* spp. (unidentified, from northern Florida) with shortleaf pine.

The widespread occurrence of *Clitocybe laccata* and the results of these tests indicate that this symbiont probably is an important mycorrhiza-former in pine stands of the Southeast. Sporophores have been found particularly abundant and widespread in slash and longleaf pine stands of northern Florida, where fresh specimens were collected throughout winter when other Hymenomycetes were lacking.

Woodruff (1933) earlier reported that *Boletus communis* was associated with mycorrhizal roots of pecan (*Carya illinoensis* (Wangenh.) K. Koch). Cultures of this Hymenomycete used in the present study were grown from sporophores collected beneath yard pecans in Athens, Georgia, where they grow in abundance from April to November during moist weather or when the lawn is frequently watered. Sporophores of *B. communis* have been collected only infrequently by the authors in forest stands of the Southeast. It is probable that this fungus is not important as a mycorrhiza-former in the pine stands of this region.

In the course of this work, two fungi, *Collybia radicata* (Fr.) Quél. and *Boletus communis*, produced sporophores in culture. Several test tube cultures of *C. radicata* growing at 25° C in the absence of light on Hagem's medium fruited after 6 to 8 months. The caps were about 1 cm in diameter, well formed, and discharged viable spores. The stipes were from 7 to 10 cm in length.

Fructification of *Boletus communis* occurred while in association with the roots of a 2-month-old slash pine seedling growing on vermiculite wetted with Melin-Norkrans' solution in a 2-liter flask. Two small, well-developed sporophores, one 1.0 cm and the other 1.5 cm tall, rose from the surface of the medium (Fig. 2A). The flask was later broken open and rhizomorphs extending from the fruiting bodies traced directly to mycorrhizal roots of the seedling (Fig. 2B).

Summary

The pure culture technique for mycorrhizal synthesis was employed to identify fungi producing mycorrhizae in association with roots of shortleaf, loblolly, slash, and longleaf pine. Fourteen species of Hymenomycetes were tested with one or all four species of pine. Mycorrhizal synthesis has been demonstrated for eight of these.

Clitocybe laccata was found to produce mycorrhizae with roots of all four pines: shortleaf, loblolly, slash, and longleaf. These results and the widespread occurrence of *C. laccata* in the Southeast suggest that this fungus is an important mycorrhiza-former in the pine stands of this region.

The fungus, *Boletus communis*, commonly associated with mycorrhizal formation with pecan was found also to produce well developed ectotrophic mycorrhizae with roots of both loblolly and slash pine.

Two cases of fruiting by test fungi in culture are reported. One, *Collybia radicata*, produced well-formed fructifications on Hagem's medium. The other, *Boletus communis*, developed sporophores when grown in association with roots of a slash pine seedling.

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